INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: PHARMACEUTICAL COMPOSITIONS CONTAINING ANTIBODY–ENZYME CONJUGATES IN COMBINATION WITH PRODRUGS

(57) Abstract

Prodrugs which can be activated by enzymes, are formulated for sequential administration, with enzyme conjugates. Either or each component comprises a polymeric carrier which allows it to be directed preferentially to the target tissue. A new polymer–prodrug conjugate is cleavable by Cathepsin B or other thiol–dependent protease. The invention is of particular utility for targeting solid tumours.

PHARMACEUTICAL COMPOSITIONS CONTAINING ANTIBODY-ENZYME CONJUGATES IN COMBINATION WITH PRODRUGS

Field of the Invention

The present invention relates to compositions and kits for use in enzyme-prodrug therapy, in which the enzyme is
5   conjugated to a carrier.

Background of the Invention

The chemotherapy of neoplastic disease is often compromised by adverse systemic toxicity which limits the dose of drug that can be administered, or is limited by the
10   appearance of multidrug resistance. Various strategies have been explored to improve drug targeting and to increase drug concentration in the tumour to a level that should overcome clinically relevant drug resistance.

Prodrugs have been used for many years in medicine to
15   treat a variety of disorders. They can offer improved solubility, improved pharmacokinetics and tissue distribution, avoidance of unfavourable metabolism, selective organ effects and specific tumour toxicity. In order to use prodrugs as anticancer agents, the tumour must
20   have a high level of the enzyme that activates the prodrug, or a foreign enzyme must be delivered, and no activating enzyme must be present in normal tissues. The prodrug must be innocuous and pharmacodynamically inert and be a substrate for the enzyme with favourable K_m and V_{max}
25   values.

The concepts of Antibody Directed Enzyme Prodrug Therapy (ADEPT) (refs. 1,2) and Gene/Viral Directed Enzyme Prodrug Therapy (G/VDEPT) (ref. 3) using either antibody conjugates or a retroviral vector to deliver an enzyme to
30   a tumour, are already well established. In ADEPT, a foreign enzyme which metabolises substrates not normally metabolised by mammalian cells is linked chemically to a tumour-specific or tumour-associated antibody; the antibody conjugate is injected intravenously and binds strongly to
35   the tumour by recognising the tumour-associated antigen. In VDEPT, the retroviral vector is designed to carry a gene expressing a preselected enzyme that can be delivered

selectively to tumour or be under the control of a tumour-
specific promoter. The foreign enzyme is then used to
activate a carefully designed low molecular weight prodrug.
Animal models and pilot human studies have proven that it
5   is possible to deliver selectively to a solid tumour an
activating enzyme such as carboxypeptidase G2, penicillin
amidase, β-lactamase, β-glucuronidase, cytosine deaminase,
nitroreductase or alkaline phosphatase (refs. 4,5).

Both approaches have a number of inherent limitations.
10   In the case of ADEPT, these include the immunogenicity of
the antibody-enzyme conjugate, the need to tailor each
conjugate to target antigen present on the tumour, the
difficulty in optimisation of the dosing schedule, and the
need to use a clearing antibody in the case of ADEPT (ref.
15   6). In the case of VDEPT, there are the inherent dangers
associated with a viral vector, the potential lack of
specificity of enzyme expression in the tumour due to
problems of delivering the gene specifically to cancer
cells, and the difficulty of evaluating the duration and
20   reproducibility of enzyme expression on a patient basis,
leading to difficulties in optimising the schedule of
prodrug follow-up.

In recent years, there has been a great deal of
investigation of polymers as carriers of anticancer drugs
25   (reviewed in ref. 7). The basis for much of this work is
that attachment of toxic drugs to high molecular weight
carriers can lead to reduction in systemic toxicity, longer
retention time in the body, alterations in biological
distribution, improvements in therapeutic efficacy and
30   site-specific passive capture through the enhanced
permeability and retention (EPR) effect. The EPR effect
results from enhanced permeability of macromolecules or
small particles within the tumour neovasculature, due to
the leakiness of its discontinuous endothelium. In
35   addition to the tumour angiogenesis (hypervasculature) and
irregular and incompleteness of vascular networks, the
attendant lack of lymphatic drainage promotes accumulation

of macromolecules that extravasate (ref. 8). This effect
is observed in many solid tumours for macromolecular agents
and lipids. The enhanced vascular permeability will
support the great demand of nutrients and oxygen for the
5   rapid growth of the tumour. Unless specifically addressed
for tumour cell uptake by receptor-medicated endocytosis,
polymers entering the intratumoural environment are taken
up relatively slowly by fluid-phase pinocytosis.

Many polymer-based anticancer agents have now entered
10   the clinic or are passing through clinical trials; each has
proven the concept compared to the native drug. For
instance, N-(2-hydroxypropyl)methacrylamide (HPMA)
copolymer-doxorubicin conjugates have already shown promise
in early clinical trial (refs. 9,10). Moreover, residual
15   HPMA copolymer conjugate which does not permeate in to the
tumour but remains in the circulation is rapidly excreted,
giving a high tumour:blood ratio (ref. 11).

In most cases, release of active anticancer drug from
the polymer support is mediated by simple aqueous
20   hydrolysis or by proteolytic or esterase enzymes (refs.
12,13). Since the conditions for these reactions are not
necessarily confined to tumour tissues, some non-specific
drug release is inevitable.

Conjugation of water-soluble polymers to
25   pharmacologically-active proteins such as enzymes, toxins,
immunoglobulins, cytoxins or allergens can be used t
reduce the proteolytic degradation of such proteins,
improve their biological efficacy and prolong plasma
elimination, as well as reduce protein immunogenicity. For
30   instance, an HPMA copolymer-asparaginase conjugate has been
used to treat leukaemia. The conjugation of the enzyme to
the polymer has been shown to increase the circulation time
of the enzyme, where its activity in depriving leukaemic
cells of asparagine is useful.

35   Summary of the Invention

According to the present invention, a product or kit
comprises two components, i.e. two pharmaceutical

4

compositions that are arranged or otherwise adapted for
sequential administration to a human or animal. The first
component is an enzyme conjugate, e.g. a composition that
comprises a pharmaceutically-acceptable excipient and an
5   enzyme conjugate. The enzyme conjugate may consist of an
enzyme covalently bound to a polymeric or other carrier
such that the enzyme conjugate retains its enzyme activity.
The second component is a prodrug, e.g. a composition that
comprises a pharmaceutically-acceptable excipient and a
10   prodrug. The prodrug is typically substantially inactive
(in terms of drug activity) but capable of being activated
by the enzyme.

It is to be noted that unless the context specifically
refers to the order of administration of the product,
15   reference herein to "first" and "second" compositions does
not imply any specific order of administration.

The two components of the kit are used in a treatment
regimen similar to ADEPT, except that the enzyme conjugate
is administered in place of the antibody-enzyme conjugate.
20   One significant difference is that the two components can
be administered, in use of the present invention, in either
order. For instance, prodrug-containing composition may be
given first. The present invention can avoid the problems
with ADEPT, resulting from the immunogenicity of the
25   antibody-enzyme conjugate. Furthermore, although a polymer
carrier is not specifically targeted to antigenic sites of
the tumour cell surface, it is nevertheless expected to
preferentially accumulate in solid tumours through the EPR
effect. It has been observed that ADEPT is surprisingly
30   non-specific in vivo, and it is believed that the polymer-
enzyme conjugate is likely to exhibit preferential
accumulation as significant as that exhibited in ADEPT.

Description of the Drawings

Figure 1 shows the reaction mechanism of drug release
35   from cephalosporin-linked prodrug by β-lactamase;

Figure 2 shows th reactions of Examples 1 and 2;

5

Figure 3 is a graph showing spectra following the reaction mixture of HPMA-Gly-Gly-ONp and β-lactamase;

Figure 4 is a graph showing the degradation of benzylpenicillin by β-lactamase and by HPMA-β-lactamase
5   conjugate;

Figure 5 is a bar chart showing free and conjugated radiolabelled β-lactamase in blood of C57 mice bearing B16F10 melanoma;

Figure 6 is a bar chart showing tumour accumulation of
10   free and conjugated radiolabelled β-lactamase in C57 mice bearing B16F10 melanoma;

Figure 7 shows the structure of the prodrug PK1, cleavable by cathepsin B;

Figure 8 is a graph showing the release of doxorubicin
15   from HPMA-Gly-Phe-Leu-Gly-dox by free and conjugated cathepsin-B and without;

Figure 9 is a bar chart showing the release of doxorubicin from PK1 in C57 mice bearing B16F10 melanoma by free and conjugated cathepsin-B; and

20   Figure 10 is a graph showing doxorubicin release from PK1 after IV administration of HPMA copolymer-cathepsin-B to C57 black male mice bearing B16F10 murine melanoma.

Description of the Invention

It is preferred that the molecular weight of the
25   enzyme conjugate should be tailored to optimise the accumulation of the conjugate in the tumour. Thus the molecular weight should be sufficiently high that the conjugate does not pass through normal endothelium, but passes into the tumour through the leaky neovasculature and
30   is accumulated by resisting removal therefrom. The molecular weight should be sufficiently low, however, for removal of residual non-tumour-accumulated conjugate through the kidneys. The total molecular weight of the conjugate should preferably be less than 1000 kD, more
35   preferably less that 100 kD, and is preferably at least 20 kD.

6

The carrier of the enzyme conjugate is preferably a polymer, e.g. a water-soluble polymer to which a covalent bond may be formed with the enzyme. The bond between the polymer and the enzyme should allow retention of the
5 enzyme's activity for the conjugate. The polymer may thus be bound through a spacer group allowing a distance between the polymer backbone and the enzyme, avoiding steric hindrance of the enzyme's active site. To avoid degradation, it may be preferred to use a polymer having a
10 molecular weight of at least 30 kD.

In the enzyme conjugate, each enzyme molecule may be bound to a molecule of polymer through one or more links, preferably through a single covalent link. Furthermore, each polymer molecule may on average have fewer than one
15 enzyme molecule bound to it. Preferably, however, the average number of enzyme molecules bound to a polymer molecule is at least one, more preferably 1 to 5. The preferred ratio depends upon the molecular weight of the enzyme, the molecular weight of the polymer and the optimum
20 total molecular weight of the conjugate for accumulation of the conjugate in a tumour by the EPR effect.

Suitable polymers which have been used as carriers and of which conjugates appear to accumulate by the EPR effect, include polyethylene glycol, ethylene glycol copolymers,
25 dextrin, polymers and copolymers of hydroxylalkyl (meth)acrylamide, for instance hydroxypropyl methacrylamide, and copolymers of styrene and maleic anhydride. Other polymers which may be used include polygalacturonic acid, copolymers of hydroxyalkyl
30 (meth)acrylate, for instance N-phenylpyrrolidone, poly(L-glutamic acid hydroxyethyl-L-glutamine), poly(α-malic acid), polyaspartic acid-PEG copolymers, poly-L-lysine and copolymers of polyethyleneimine.

Whilst the enzyme-polymer conjugate may rely for its
35 localisation at a solid tumour primarily upon EPR, it may be desirable to attach ligands allowing active targeting. For instance, it may be desirable to attach galactose

ligands to target the conjugate to liver. Alternatively,
melanocyte-stimulating hormone may be bound to the polymer,
as a ligand for melanoma. If desired, an antibody may be
joined to the polymer as a ligand, the antibody being
5   directed to specific antigenic sites on the target tissue
of interest. Whilst the use of such an antibody-polymer-
enzyme conjugate may have some advantages over the
antibody-enzyme conjugate used in the normal ADEPT system
in terms of reduced immunogenicity, the present invention
10   preferably avoids the use of active targeting ligands for
the enzyme conjugate.

    The enzyme conjugate includes functional enzyme. The
conjugate preferably retains a significant level of enzyme
activity. The level may be reduced as compared to the
15   native enzyme, provided that significant useful activity is
retained.

    The prodrug used in the invention may be a relatively
low molecular weight compound. The action of the enzyme on
the prodrug may not significantly change the molecular
20   weight of the compound. A useful summary of suitable
prodrugs and the enzymes which activate the prodrugs is
given in Table 1 of reference 5, a review article citing
many references in which full details of the prodrugs and
their use are described. Thus the enzyme may be selected
25   from DT diaphorase, plasmin, carboxypeptidase G2, thymidine
kinase (viral), cytosine deaminase, glucose oxidase,
xanthine oxidase, carboxypeptidase A, α-galactosidase,
β-glucosidase, azoreductase, γ-glutamyltransferase,
β-glucuronidase, β-lactamase, alkaline phosphatase,
30   aminopeptidase, penicillin amidase and nitroreductase.

    One preferred enzyme is β-lactamase. The β-lactamases
are a group of enzymes of varying specificity but all are
capable of hydrolysing β-lactams to a substituted β-amino
acid. Some act more readily on penicillins, while others
35   have greater activity against cephalosporins. β-Lactamase
activity is not endogenous to the mammalian systems and it

8

is therefore subject to minimal interference from
inhibitors, enzyme substrates or endogenous enzyme systems.

The synthesis of a range of cephalosporin derivatives
of various mustards (ref. 14) and doxorubicin (ref. 15) has
5   been described and these are relatively good substrates for
the enzyme. The spacer between the polymer and the
anticancer drug will contain a β-lactam ring in the form of
a penicillin or cephalosporin. β-Lactamase cleaves the
lactam ring and after a chemical rearrangement (shown in
10   Fig. 1), the drug is produced. Many cytotoxic agents can
be attached, provided that they have NH$_2$ or OH groups that
can be substituted and thus act as prodrugs. The rate of
the hydrolysis is measured *in vitro* by the change in
absorbance.

15   Another preferred class of enzyme is protease/
peptidase. Thiol-dependent proteases such as cathepsin B
are particularly preferred.

The prodrug should be substantially inactive in terms
drug activity. Thus the activated drug should preferably
20   have an activity which is at least 100 times that of the
prodrug, most preferably at least 1000 times the activity.

The drug may be selected from anti-tumour agents,
antibiotics, antimetabolites, alkylating agents, alkaloids
and microtubule inhibitors, and signal transduction
25   modifiers. Preferably it is a cytotoxic agent, as the
present invention has its primary utility in treatment of
cancers. Thus the drug may be any known cytotoxic agent
capable of being provided in the form of a prodrug for
enzymic activation.

30   A suitable list of drugs and their prospective
prodrugs, as well as the respective activating enzymes is
given in reference 5, Table 1. Thus the drug may be
selected from:

5-(Azurudub-1-yl)-4-dihydroxylamino-2-nitrobenzamide
35   Phenylenediamine mustard
Benzoic acid mustards (various)
Gangcyclovir triphosphate

Adenine arabinonucleoside triphosphate (araATP)

5-Fluorouracil

Hydrogen peroxide

Superoxide, hydrogen peroxide

Methotrexate

Cyanide

Phenylenediamine mustards (various)

Phenylendiamine mustard

Phenol Mustard

Anthracyclines e.g. Epirubicin, Doxorubicin and Daunorubicin

4-Desacetylvinblastine-3-carboxhydrazide

Nitrogen mustards (various)

Mitomycin alcohol

Etoposide

Palytoxin

Melphalan

5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide

Actinomycin D, mitomycin C

Taxanes, such as taxol and taxotere

Topoisomerase inhibitors such as camptothecin and topotecan.

Preferably the prodrug comprises a carrier to which the drug is conjugated. In some circumstances, it may be desirable for the enzymic activation of the prodrug not to be accompanied by release of activated drug from the polymer conjugate. Thus an activated drug-polym r conjugate may have polymer bound to drug moieties through linker moieties which are not cleaved by the enzyme, which allow the drug's activity to be retained. Alternatively, the activation of the prodrug through reaction of the enzyme may take place independently of cleavage of the drug moiety from the polymer conjugate, for instance through activity of a separate enzyme on the linker joining th drug moiety to the polymer.

It is, however, preferred for activation of a prodrug-polymer conjugate to occur by cleavage of a linker which is susceptible to cleavage by the enzyme.

Thus the linker may be a cephalosporin linker, which
5   is cleaved by β-lactamase as described in reference 15. Preferably, however, the linker is a peptide moiety and the enzyme is a peptidase enzyme for which the peptide linker is the substrate.

In another embodiment of the invention, a prodrug
10   comprises a conjugate of a polymer and a drug moiety joined together by a peptidyl linker, and an enzyme conjugate comprises an enzyme bound to a carrier moiety. The conjugate and the prodrug are components of a novel kit comprising two compositions, arranged for sequential
15   administration, each containing one of the aforementioned conjugates.

The conjugate of polymer and drug moiety preferably has a molecular weight in the range 5 kD to 1000 kD, more preferably in the range 20 kD to 100 kD.

20   In this aspect of the invention, the enzyme conjugate may be a polymer-enzyme conjugate as used in the first aspect of the invention. It may also be an antibody-enzyme conjugate, for instance as used in ADEPT. Alternatively, the carrier may be another type of molecule capable of
25   being actively targeted to desired target cells, or to which other proteins or components may specifically bind. For instance, the carrier may comprise a hormone or other specific moiety capable of specific binding to a carrier present in the circulation or to receptors on cell
30   surfaces.

The enzyme used in this aspect of the invention is a peptidase enzyme capable of cleavage of the peptidyl linker of the prodrug conjugate. Cleavage of the linker preferably results in release of active drug.
35   Alternatively, the enzymic reaction product may further react through subsequent steps to generate the active drug in situ. Preferably, the peptidase enzyme is a specific

enzyme which cleaves specific peptide sequences only, and
is not a non-specific peptidase.

The peptidyl linker comprises sufficient amino acid
units to allow specific binding and cleavage by peptidase
5   enzyme. Preferably, the linker is at least two amino acids
long, more preferably at least three amino acids long, for
instance four or more amino acids long.

In this aspect of the invention, again, the drug is
preferably a cytotoxic drug. It is convenient for the
10 peptidyl linker to be cleavable by enzymes which are
present at relatively high levels in tumours as compared to
tissue. A suitable enzyme is a thiol-dependent protease,
for instance cathepsin-B. A specific substrate for
cathepsin-B is a Gly-Phe-Leu-Gly linker.
15   Conjugation of polymer, linker and drug may be carried
out using conventional chemical and biochemical techniques.
For instance, pendant groups on the polymer may react with
preformed peptide compounds and the polymer with peptide
side-chains may subsequently be reacted with drug
20 molecules. Alternatively, polymerisable peptide compounds
are readily available and may be copolymerised with other
monomers to form a polymer with pendant peptide groups, to
which a drug compound may be reacted. The synthesis of the
Gly-Phe-Leu-Gly-doxorubicin-HPMA conjugate is described in
25 reference 16. It is shown to have reduced toxicity
compared to free doxorubicin and improved anti-tumour
activity in reference 17.

A further aspect of the present invention lies in the
use of thiol-dependent proteases as the enzyme component of
30 enzyme/prodrug therapy. A novel conjugate consists of a
thiol-dependent protease covalently bound to a carrier,
said conjugate retaining thiol-dependent protease activity.
The enzyme is preferably cathepsin-B. The conjugate may be
provided as part of a pharmaceutical composition or kit.
35   In this, third aspect of the invention, the carrier
may, as for the second aspect, be an antibody or other
active targeting component, but is preferably a polymer.

12

Whilst it is preferred for a thiol-dependent protease-polymer conjugate not to be actively targeted, it may be possible to provide the polymer with active ligands such as antibodies and other specific binding components.

5   A product or kit comprises the novel conjugate and a prodrug comprising a drug bound to a carrier via a linker cleavable by the thiol-dependent protease.

In each aspect of the invention, the components may be used in a method of treatment. The treatment will be that

10   associated with the drug. The amount of each component that should be administered will be dependent on the nature of the condition being treated and the particular drug that is used, as will be readily evident to one of ordinary skill in the art. Typical amounts and dosage regimes may

15   be the same as or similar to those for related procedures, and can then be adjusted, if necessary.

In each aspect of the invention, the components may be administered to a patient in either order. It is preferred that the first composition to be administered is selected

20   such that it is accumulated in tumour by EPR. Preferably, the component in the first composition to be administered is capable of being cleared normally from the circulation by the kidneys after a period of less than, for instance, 12 hours, preferably less than 6 hours (the circulation

25   half-life). It is particularly convenient for the prodrug conjugate to be administered first, to achieve rapid accumulation in the tumour and subsequent clearance from the circulation of the potentially toxic prodrug by excretion through the kidneys. This is a significant

30   improvement over the ADEPT system where the enzyme has to administered first.

Where the first administered component is not cleared from the circulation adequately, it may be appropriate to clear the component from the circulation by administering

35   a clearing component. The first component to b administered may be provided with a ligand which is adapted for binding to an anti-ligand. This component can thus be

cleared from the circulation by a second, clearing
component which comprises an anti-ligand and a moiety which
allows the clearing component to be adapted to be cleared
from the circulation.

5   Preferably the first component to be administered in
the novel method is the prodrug. It should therefore be
adapted to optimise its EPR and hence tumour accumulation.
Whilst the enzyme conjugate, which is administered second,
in such preferred embodiments, is advantageously directed
10   preferentially to the tumour, since the enzyme conjugate is
generally substantially non-toxic, it is less important
that the enzyme conjugate be adapted to optimise the EPR
effect.   The circulation time of the enzyme conjugate
should be sufficiently long for activation at the desired
15   site of substantially all the prodrug. The difference in
time between the administration of the two compositions may
be hours or even days. Controlling the molecular weight of
the respective conjugates allows a degree of control of the
optimum administration protocol.

20   An advantage of the third aspect of the present
invention is that the prodrug, when preferentially
accumulated in the tumour, will be activated intra-
cellularly by endogenous thiol-dependent protease, present
in increased levels inside the cells in tumours, and
25   extracellularly by the thiol-dependent protease-carrier
conjugate. This provides active drug outside cells which
may be transported into cells which do not otherwise
contain activated drug.

          The invention is further illustrated in the following
30   Examples, with reference to Figs. 2 etc. of the
accompanying drawings.

Example 1: HPMA-β-lactamase conjugate

          β-Lactamase (Sigma; ~28kDa) was attached to the
polymeric   carrier   N-(2-hydroxypropyl)methacrylamide-
35   Glycine-Glycine-p-nitrophenol (HPMA-Gly-Gly-ONp; Polymer
Laboratories; ~30kDa) via the nonspecific aminolytic

reaction of the ONp groups at controlled pH. The reaction
is shown in Fig. 2. The following steps were conducted:

The polymer bearing in the side-chain p-nitrophenol
(ONp) groups was dissolved in double-distilled water (4
5   mg/ml), and this β-lactamase was dissolved in 0.05M
phosphate buffer, pH 7.2 (2 mg/ml), and this solution was
added to the polymer solution at 4°C under stirring.

The reaction mixture was stirred in the dark at pH 7.2
for 30 min. The pH was carefully raised over 4 h by adding
10 saturated sodium tetraborate buffer up to pH 8.5 (to
prevent enzyme denaturation), and the reaction mixture
stirred for another 4 h. The reaction was finished by
adding 1-amino-2-propanol (1/2 the equivalent related to
the original ONp groups), to remove unreacted ONp groups.
15 The solution was acidified to pH 7.2.

To remove free polymer and free enzyme, the conjugate
was purified by Spectra/POR®CE (Cellulose Ester) sterile
DispoDialyzer MW cut-off 50 kDa for separation.

The conjugation reaction was followed by UV
20 spectrophotometry, showing the release of p-nitrophenol
groups from the HPMA-copolymer. ONp bound to HPMA absorbs
at λmax 270 nm while free ONp absorbs at 400 nm. The
spectra are shown in Fig. 3.

Proteins reduce alkaline Cu(II) to Cu(I) in a
25 concentration-dependent manner. Bicinchoninic acid is a
highly specific chromogenic reagent for Cu(I), forming a
purple complex with an absorbance maximum at 562 nm. The
absorbance is directly proportional to protein
concentration.
30 The results of the bicinchoninic assay are shown in
the following table:

BSA µg per 20µl	BSA mean abs	BSA ±SD	beta-lactamase µg/20µl	beta-lactamase mean abs	beta-lactamase ±SD	conjug. µg per 20µl	conjug. mean abs	conjug.
0	0	0	80	0.252	0.011	4.113	0.0293	0.0037
2	0.05	0.003	40	0.146	0.004	43.774	0.1486	0.0049
4	0.1	0.002	20	0.853	0.002			
8	0.17	0.018	10	0.045	0.001			
12	0.23	0.03	6.67	0.033	0.002			
16	0.31	0.032	5	0.026	0.003			
20	0.37	0.004						
24	0.45	0.042						

- 4.1µg beta-lactamase in 20µl sample of conjugate after separation >50kDa (0.21mg/ml).
- 43.8µg beta-lactamase in 20µl sample before separation (2.2mg/ml).

The yield is (205.7/1000) x 100 = 20.57%

SDS-PAGE was done in order to show that the release of p-nitrophenol groups was due to aminolysis and not hydrolysis by the phosphate buffer. The free β-lactamase was compared to the bound one relating to molecular weight markers. Unreacted HPMA, as expected from a polymer, gave no band. The free β-lactamase gave two bands coincident with a 30 kDa molecular weight marker due to the two types of enzyme present. The HPMA copolymer-β-lactamase conjugate gave a band corresponding to 60 kDa; after purification, no free enzyme was detected.

In an enzyme activity assay, 10 units/ml β-lactamase (free or conjugated) in phosphate buffer (0.1M, pH 7.0) were added to benzylpenicillin solution (1 mM) and the decreasing UV absorbance at 240 nm was followed with time.

The activity assay was done using the same amount of enzyme in the free enzyme activity test and in the conjugated enzyme activity test, i.e. 10 units per ml. The activity was tested on free benzylpenicillin as a representative substrate. Results showed that the β-lactamase retained enzyme activity after polymer

16

conjugation; although reduced, it was active enough to cleave the spacer in the final form of PDEPT. The results are shown in Fig. 4.

5   Michaelis-Menten constants were calculated from the above assays based on the use of free enzyme compared to the conjugated one, in constant concentration (10 units/ml), on different concentrations of benzylpenicillin (1.0 mM, 0.9 mM, 0.75 mM, 0.5 mM, 0.33 mM). The change in absorbance was calculated over time until reaching a

10   plateau. The linear part of the degradation of the different substrate concentrations was plotted and from the slope Vo was calculated and the Lineweaver-Burk graph was plotted.

For the free β-lactamase, Vmax is 0.77 nM/s/unit and

15   Km is 0.22. For the conjugate, Vmax is 0.20 nM/s/unit and Km is 0.07.

The conjugate of Example 1 is suitable for use in combination with a suitable prodrug.

The *in vivo* biodistribution of HPMA copolymer-enzyme

20   compounds was tested in animal experiments conducted according to the UKCCCR (United Kingdom Co-ordinating Committee on Cancer Research) Guidelines. A B16F10 murine melanoma s.c. model was used.

Male C57BL/6J mice were inoculated with 10^5 viable

25   B16F10 cells subcutaneously (s.c.). The tumour was allowed to establish until the area was approximately 50-70 mm^2 as measured by the product of two orthogonal diameters. 1 x 10^6 counts were diluted into 0.5 ml in 0.9% NaCl. The pH was then neutralised with saturated NaOH and the final

30   volume was then made up to 1.0 ml. Of this sample, 100 μl were withdrawn using a 1 ml syringe and 12 gauge needle and counted as a measure of injected dose.

100 μl (5 x 10^5 CPM) of free or conjugated β-lactamase were injected into the tail vein of C57BL/6J mice (3

35   replicates per time point). The mice were then placed in a metabolism cage and sacrificed at different time points up to 48 hours. The following tissue samples were

17

dissected: tumour, liver, kidneys, lung, heart, spleen and faeces, urine and blood were collected. Samples were weighed and homogenised in PBS. Samples (1 ml) were then counted in a γ-counter in replicates of 3 (three samples

5   per organ per mouse). The results were then expressed as % dose injected for each organ or % dose recovered for each organ. The blood volume of the mouse was calculated assuming 5.6 ml blood/100 g mouse. The results are shown in Figures 5 and 6.

10   **Example 2: HPMA-Cathepsin B conjugate**

Cathepsin-B (Sigma; ~28 kDa) was attached to the polymeric carrier (HPMA-Gly-Gly-ONp; ~30 kDa) via the nonspecific aminolytic reaction of the ONp groups at controlled pH. The reaction is shown in Fig. 2. The same

15   steps were conducted as in Example 1, except that the polymer was dissolved in double-distilled water at 1 mg/ml and cathepsin-B was used instead of β-lactamase.

The conjugation reaction was again followed by UV spectrophotometry.

20   The free cathepsin-B was compared to the bound one relating to molecular weight markers, by SDS-PAGE. Unreacted HPMA, as expected from a polymer, gave no band. The free cathepsin-B gave bands coincident with 30 kDa and 90 kDa molecular weight marker due to the different chains

25   of enzyme present. The HPMA copolymer-cathepsin-B conjugate gave a band corresponding to 60 kDa and 97 kDa (due to cross-linking). After purification no free enzyme was detected.

Activity of free or conjugated cathepsin-B was assayed

30   by measuring the release of p-nitroaniline (NAp) at 410 nm from the tripeptide Bz-Phe-Val-Arg-NAp during incubation at 37°C in citrate buffer (0.2 M, pH 5.5) containing EDTA (10 mM) and reduced glutathione (50 mM).

The cathepsin-B conjugate of Example 2 is suitable for

35   use in combination with a suitable prodrug such as HPMA-Gly-Phe-Leu-Gly-doxorubicin (PK1; see Fig. 7).

18

The activity of cathepsin-B (free or conjugated) on
the high molecular weight substrate PK1 was assessed by
HPLC. Incubation of PK1 with cathepsin-B was carried out
at 37°C in a final volume of 1 ml consisting of 400 μl 1
5   mg/ml of PK1 in citrate buffer (pH 5.5, 0.2 M), 100 μl EDTA
solution in buffer (10 mM), 100 μl reduced glutathione (GSH
50 mM) and 400 μl HPMA-cathepsin-B or free cathepsin-B in
buffer (equivalent to cathepsin-B concentration present in
the conjugate - 1 mg/ml). Tubes containing PK1 without
10   enzymes were also prepared, to assess the levels of
hydrolysis, as controls. 100 μl samples were taken at
various times, immediately frozen in liquid nitrogen and
stored frozen in the dark until processed by HPLC. Then
the samples were mixed with 100 ng daunomycin (DNM), as an
15   internal standard, ammonium formate buffer (pH 8.5 and
extraction mixture (chloroform:propan-2-ol). Tubes were
centrifuged, the aqueous layer was carefully removed and
the organic fraction evaporated to dryness using the Techne
nitrogen system operated at no more than 13.8 kPa (2
20   lbf/in^2) of N_2(g). Evaporated samples were redissolved in
methanol prior to analysis. The release of free
doxorubicin from PK1 *in vitro* is shown in Fig. 8.

Evaluation *in vivo* was conducted using the B16F10
murine melanoma model. Male C57BL/6J mice were inoculated
25   with 10^5 viable B16F10 cells subcutaneously (s.c.). The
tumour was allowed to establish until the area was
approximately 50-70 mm^2 as measured by the product of tw
orthogonal diameters. Animals were injected i.v. with PK1
(5 mg/kg and 10 mg/kg equivalent of doxorubicin) or PK1 (5
30   mg/kg and 10 mg/kg equivalent of doxorubicin) followed
after 5 hours by free cathepsin-B (3.63 mg/kg) or HPMA-
copolymer-Gly-Gly-cathepsin-B (3.63 mg/kg equivalent of
cathepsin-B) and sacrificed at different time points up to
48 hours. The following tissue samples were dissected:
35   tumour, liver, kidneys, lung, heart, spleen and urine and
blood were collected. Samples were weighed, homogenised in
PBS, and mixed with 100 ng daunomycin (DNM), as an internal

standard, ammonium formate buffer (pH 8/5) and extraction
mixture (chloroform:propan-2-ol). Tubes were centrifuged,
the aqueous layer was carefully removed and the organic
fraction evaporated to dryness using the Techne nitrogen
5   system operated at no more than 13.8 kPa (2 lbf/in^2) of
N$_2$(g). Evaporated samples were redissolved in methanol
prior to HPLC analysis. The results, comparing the release
of doxorubicin from the polymer conjugate with and without
the enzyme, are shown in Figs 9 and 10.

References

1. Bagshawe, Br. J. Cancer 56:531-532 (1987).

2. Senter, The FASEB Journal 4:188-193 (1990).

3. Ram et al, Cancer Res. 53:83-88 (1993).

4. Knox and Connors, Clinical Immunotherapy 3:136-153 (1995).

5. Melton et al, Drugs of The Future 21(2):167-181 (1996).

6. Bagshawe and Begent, Advanced Drug Delivery Reviews, 22:365-367 (1996).

7. Duncan et al, S. T. P. Pharma Sci. 6(4):237-263 (1996).

8. Maeda and Matsumura, CRC Critical Rev. Therap. Drug Carrier Sys. 6:193-210 (1989).

9. Rihova and Kopecek, J. Controlled Rel. 2:289-310 (1985).

10. Flanagan et al, J. Bioactive and Compatible Polymers 5:151-166 (1990).

11. Seymor et al, Eur. J. Cancer (May 1995).

12. Maeda et al, Bioconj. Chem. 3:351-362 (1992).

13. Takakura and Hashida, Crit. Rev. Onco. Hematol. 18:207-231 (1995).

14. Svensson et al, Bioconjug. Chem. 3:176-181 (1992).

15. Senter et al, Bioconjug. Chem. 6:389-394 (1995).

16. Seymor et al, Brit. J. Cancer, 70:636-641 (1994).

17. Duncan et al, J. Controlled Rel. 3:175-210 (1992).

CLAIMS

1.   A product comprising, as a combined preparation for sequential administration, in drug treatment, an enzyme conjugate and a prodrug, the enzyme conjugate consisting of a functional enzyme covalently bound to a polymeric carrier, and the prodrug being substantially inactive (in terms of drug activity) but capable of being activated by the enzyme.

2.   A product according to claim 1, in which the prodrug comprises a carrier to which the drug is covalently conjugated.

3.   A product according to claim 2, in which the carrier of the prodrug is a polymer.

4.   A product according to claim 3, in which the enzyme is a protease and the drug is conjugated to the polymeric carrier by a peptidyl linker which is cleavable by a protease enzyme.

5.   A product comprising, as a combined preparation for sequential administration, in drug treatment, an enzyme conjugate and a prodrug, the prodrug comprising a drug bound to a polymeric carrier by a peptide linker which is cleavable by the enzyme.

6.   A product according to claim 5, in which the enzyme conjugate comprises an antibody to which the enzyme is covalently conjugated.

7.   A product according to any preceding claim, in which the enzyme is a thiol-dependent protease.

8.   A product according to any of claims 1 to 4, in which the enzyme is β-lactamase.

9.   A product comprising, as a combined preparation for sequential administration in drug treatment, an enzyme conjugate and a prodrug, the enzyme conjugate consisting of a functional thiol-dependent protease covalently bound to a carrier, and the prodrug comprising a drug bound to a carrier by a peptide linker which is a substrate for the thiol-dependent protease.

10. A product according to claim 7 or claim 9, in which the thiol-dependent protease is cathepsin-B.

11. A product according to claim 9 or claim 10, in which the or each carrier is a polymer.

12. A product according to any of claims 1 to 8 and 11, in which any polymeric carrier comprises a polymer or copolymer of a hydroxyalkyl (meth)acrylamide, preferably hydroxypropyl methacrylamide.

13. A conjugate which consists of a functional thiol-dependent protease covalently bound to a carrier.

14. A conjugate according to claim 13, in which the protease is cathepsin-B.

15. A conjugate according to claim 13 or claim 14, in which the carrier is a polymer, e.g. as in claim 12.

16. A conjugate according to any of claims 13 to 15, for use in therapy.

17. A pharmaceutical composition comprising a pharmaceutically-acceptable excipient and a conjugate according to any of claims 13 to 15.

18. Use of a prodrug as defined in any of claims 1 to 5, 9 and 12, for the manufacture of a medicament for use in therapy associated with the active drug, the therapy comprising the separate administration of an enzyme that releases the drug from the prodrug.

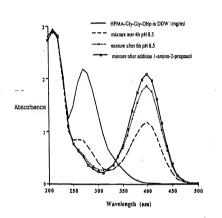19. Use according to claim 18, in which the prodrug is administered before the enzyme conjugate.

Fig. 1

$$\left[-CH_2 - \underset{\underset{\underset{\underset{\underset{CH_3}{\overset{|}{CHOH}}}{\overset{|}{CH_2}}}{\overset{|}{NH}}}{\overset{\overset{CH_3}{\overset{|}{C}}}{\underset{|}{CO}}} - \right]_{95} \left[-CH_2 - \underset{...}{\overset{...}{C}} - \right]_5$$

β–Lactamase/ Cathepsin-B

Fig. 2

SUBSTITUTE SHEET (RULE 26)

Fig. 3

Fig. 4

Fig. 5

6/10

Fig. 6

7/10
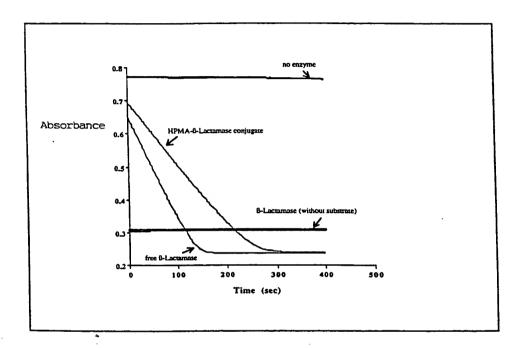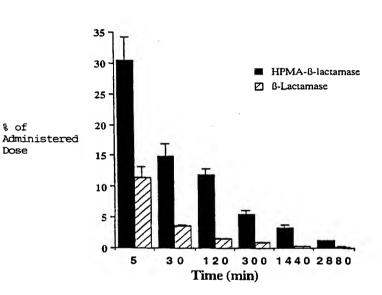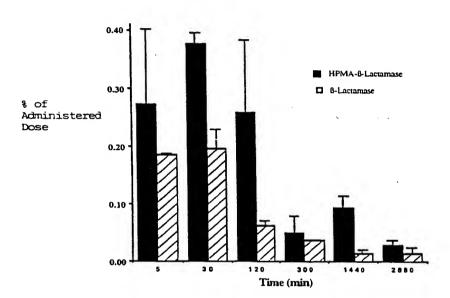

Fig. 7
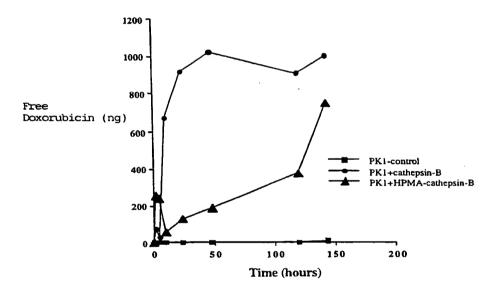
Fig. 8
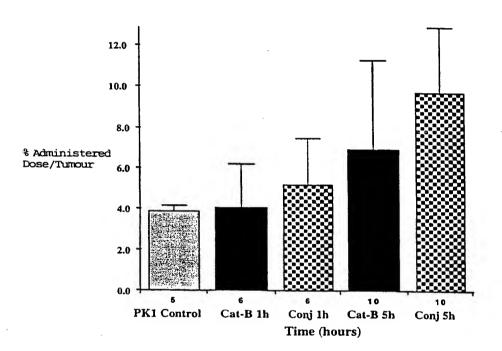
Fig. 9
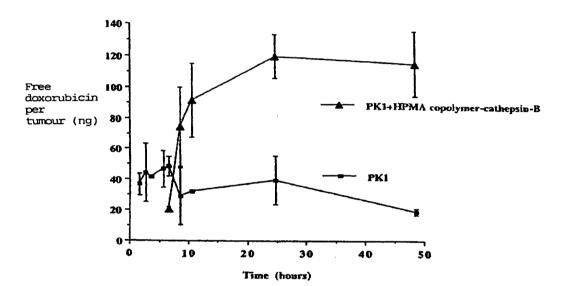
Fig. 10

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KRINICK NL: "Combination polymeric drugs as anticancer agents." DISS ABSTR INT 'B!;52(12):6525 1992, XP002049282 see abstract	¬1

A	NICHIFOR M ET AL: "Macromolecular prodrugs of 5-fluorouracil. 2: Enzymatic degradation" JOURNAL OF CONTROLLED RELEASE, 39 (1). 1996. 79-92., XP002040091 see abstract	1-19

	-/--	

| X | Further documents are listed in the continuation of box C. | | Patent family members are listed in annex. |

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 1998	05/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Leherte, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category¹	Citation of document. with indication.where appropriate. of the relevant passages	Relevant to claim No.
A	NICHIFOR M. ET AL: "Polymeric prodrugs of 5-fluorouracil" JOURNAL OF CONTROLLED RELEASE, 1997, 48/2-3 (165-178), XP002049283 NETHERLANDS see page 176, column 2 - page 177, column 1 ---	1-19
A	MELTON R G ET AL: "ANTIBODY-ENZYME CONJUGATES FOR CANCER THERAPY" JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 88, no. 3/04, 21 February 1996, pages 153-165, XP002038773 see abstract ---	1-19
A	SENTER P D ET AL: "POLY(ETHYLENE GLYCOL)-DOXURUBICIN CONJUGATES CONTAINING -LACTAMASE-SENSITIVE LINKERS" BIOCONJUGATE CHEMISTRY, vol. 6, no. 4, 1 July 1995, pages 389-394, XP000517227 cited in the application see abstract ---	1-19
A	WALLACE P M ET AL: "Selective Activation of Anticancer Prodrugs by Monoclonal Antibody-Enzyme Conjugates" METHODS AND FINDINGS IN EXPERIMENTAL AND CLINICAL PHARMACOLOGY, 16 (7). 1994. 505-512., XP002049284 see abstract ------	1-19

1